



Novel cardiac protective effects of urea: from shark to rat

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1 This study was carried out to investigate novel cardioprotective effects of urea and the underlying mechanisms. The cardiac functions under oxidative stress were evaluated using Langendorff perfused isolated heart.

2 Isolated dogfish shark hearts tolerated the oxidative stress generated by electrolysis (10 mA, 1 min) of the perfusion solution ($n=4$), and also showed normal cardiac functions during post-ischaemia reperfusion ($n=4$). The high concentration of urea (350 mM) in the heart perfusate was indispensable for maintaining the normal cardiac functions of the shark heart.

3 Urea at 3–300 mM ($n=4$ for each group) protected the isolated rat heart against both electrolysis-induced heart damage and post-ischaemia reperfusion-induced cardiac injury.

4 A concentration-dependent scavenging effect of urea (3–300 mM, $n=4$ for each group) against electrolysis-induced reactive oxygen species was also demonstrated *in vitro*.

5 Urea derivatives as hydroxyurea, dimethylurea, and thiourea had antioxidant cardioprotective effect against the electrolysis-induced cardiac dysfunction of rat heart, but were not as effective as urea in suppressing the post-ischaemia reperfusion injury.

6 Our results suggest that urea and its derivatives are potential antioxidant cardioprotective agents against oxidative stress-induced myocardium damage including the post-ischaemia reperfusion-induced injury.

Keywords: Urea; ischaemia; reperfusion; heart; oxidative stress; arrhythmia

Abbreviations: CF, coronary flow; DPD, N,N'-diethyl-p-phenylenediamine; ECG, electrocardiogram; EPS, Elasmobranch physiological solution; HR, heart beating rate; IVF, irreversible ventricular fibrillation; KH, Krebs-Henseleit; LVDP, left ventricular developed pressure; OFR, oxygen free radicals; ROS, reactive oxygen species; TMAO, trimethylamine N-oxide; VDP, ventricular developed pressure; VF, ventricular fibrillation

Introduction

Cardiac dysfunction during post-ischaemia reperfusion has been largely attributed to the detrimental effects of reactive oxygen species (ROS) (Simpson & Lucchesi, 1987; Manning, 1988). Among the possible mechanisms involved in the ROS generation, xanthine oxidase was considered to play a central role (McCord *et al.*, 1985; Thompson-Gorman & Zweier, 1990). Inhibitors of this enzyme were used to prevent ROS-induced heart injury (Manning & Hearse, 1984; Akizuki *et al.*, 1985), but side effects were observed due to either the inhibitors themselves or to the accumulated substrates of xanthine oxidase (Thompson-Gorman & Zweier, 1990). Antioxidants, such as mannitol and vitamin E (Bernier *et al.*, 1986; Opie, 1989), and ROS scavengers, such as superoxide dismutase (SOD, EC 1.15.1.1) (Meerson *et al.*, 1987; Woodward & Zakaria, 1995), have also been shown to reduce the severity of the post-ischaemia reperfusion injury. However, some properties of these agents such as cytotoxicity (Visweswaran *et al.*, 1997), prooxidant activity (Edge & Truscott, 1997), or high molecular weight in the case of SOD, limited their application as cardioprotective agents. Recently, it was reported that copper proteins such as ceruloplasmin (EC 1.16.3.1) (Atanasiu *et al.*, 1995; Mateescu *et al.*, 1995) and

serum amine oxidase (EC 1.16.3.6) (Mondovi *et al.*, 1997) also exerted cardioprotective effects against post-ischaemia reperfusion-induced heart injury. But as proteins, some difficulties of their pharmacological formulation and administration may occur.

Urea is the most common nitrogen-containing end product of protein catabolism (Wright, 1995), mainly resulting from the detoxification of the ammonia derived from amino acids deamination (Meijer *et al.*, 1990). Urea crosses cell membranes by two ways: specialized transporters or nonspecific pores (Marsh & Knepper, 1992). Urea synthesis is involved in the regulation of chronic acid-base disturbances (Atkinson, 1992). Whether the accumulated urea in the plasma in hyperuremia contributes to the uremic syndrome has been an issue of controversy (Giovannetti & Barsotti, 1975). Recently, it was found that urea stimulated gene transcription and expression (Cohen *et al.*, 1996). While some urea derivatives, such as thiourea, dimethylurea and dimethylthiourea, were recognized as potent antioxidants, urea itself was conventionally considered as a metabolic waste or at best an inert substance used as negative control in some antioxidant studies (Halliwell, 1978). However, Lukash *et al.* (1980) reported that urea, inhibiting the oxidation of divalent iron, might have an antioxidant effect protecting brain and liver tissue homogenates from peroxidation of lipids. Its inhibitory effect on NADPH-dependent lipid peroxidation in rat heart tissue was also described (Shveta & Davydov, 1994).

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Dogfish shark (*Squalus acanthias*) plasma contains a particularly high level of urea. This suggested that shark could probably be oxidative stress-resistant. In this study, the cardiac functions of the isolated dogfish shark heart under oxidative stress induced by both electrolysis and post-ischaemia reperfusion were investigated, *ex vivo*, and the results compared with those obtained from isolated rat hearts. The potencies of urea and several urea derivatives in protecting hearts from post-ischaemia reperfusion damage and in scavenging ROS were also evaluated. Furthermore, the direct ROS scavenging activity of urea was examined *in vitro*. To our knowledge, this is the first study revealing high antioxidant cardioprotective capacity of urea. This newly observed effect of urea might have broad applications towards many oxidative stress-related pathophysiological situations.

Methods

Materials

Urea, trimethylamine N-oxide (TMAO), N,N'-diethyl-p-phenylenediamine (DPD), and the other chemicals were from Sigma Chem. Co. (St-Louis, MO, U.S.A.). All chemicals were of reagent grade and used without further purification.

Evaluation of cardiac functions of isolated shark heart under the oxidative stress induced by electrolysis or post-ischaemia reperfusion

Dogfish sharks (2–4 kg, male or female) were gillnetted in Frenchman Bay (ME, U.S.A.) by the collector of the Mt. Desert Island Biological Laboratory and maintained, without feeding, in submerged wooden live cars until use (1–2 days). To evaluate cardiac functions, the sharks were pithed and heart removed, washed in iced elasmobranch physiological saline (EPS) and mounted on a modified Langendorff perfusion system. The heart of dogfish shark consists of a ventricle and an atrium. A pair of coronary arteries ramifies over the ventricle and sends small branches to the walls of the pericardial cavity. The isolated hearts were perfused at a constant pressure of 5 kPa (50 cmH₂O) at 25°C with EPS (pH 7.4) containing (in mM): NaCl 270, KCl 4, TMAO 150, urea 350, MgCl₂ 3, HEPES 10, CaCl₂ 3, Na₂SO₄ 0.5, KH₂PO₄ 0.5 and heparin (50 units ml⁻¹). The EPS was continuously gassed with a mixture of 95% O₂ and 5% CO₂. Ventricular developed pressure (VDP) was detected by a pressure sensor connected to a three-way valve, 10 cm above the aorta. Epicardial electrograms (EPI-ECG) were recorded with two electrodes positioned one on the aorta and the other on the apex of the heart. Data acquisition and analysis were accomplished using a Biopac system (Biopac System Inc., Golata, U.S.A.), including MP 100 WS acquisition units, TCI 100 amplifiers, an Acknowledge software (3.01) and universal modules (Macintosh system).

The exogenous generation of ROS was realized by placing two platinum wire electrodes in the inflow cannula: the anode at 12 cm and the cathode at 15 cm away from the entry port of the atrium (Jackson *et al.*, 1986). A direct current (DC), generated under various current intensities (10–30 mA) and different duration (1–3 min) by a Grass stimulator (Quincy, U.S.A.), was applied. A bubble trap placed above aorta prevented bubbles from getting into the heart. Endogenous ROS were generated by the reperfusion of the regional ischaemia, induced by occluding one coronary artery by ligation as previously described (Atanasiu *et al.*, 1995).

The experimental protocols were divided into two groups.

Electrolysis group The total recording time was set for 35 min (Figure 1A). The first 15 min of period served as control. Electrolysis started at the 16th min and lasted for 1, 2 or 3 min, respectively, with 10 mA DC. Alternatively, the electrolysis duration was kept constant for 1 min with different intensity of DC (10, 20 and 30 mA). The EPI-ECG and VDP were continuously recorded during the experiment.

Post-ischaemia reperfusion group The total recording time was set for 40 min (Figure 1B). The first 15 min served as control and the partial ischaemia was induced at the 16th min by occlusion of one of the two coronary arteries, and maintained for 10 min. Subsequently, the shark heart was reperfused for 15 min. During the whole process, EPI-ECG and VDP were continuously recorded.

Evaluation of cardiac functions of isolated rat heart under the oxidative stress induced by electrolysis or post-ischaemia reperfusion

Male Wistar rats (250–275 g) were anaesthetized, i.p. with Somnitol (0.1 ml 100 g⁻¹ body weight) and heparinized (500 IU intravenously). The hearts were excised, washed in cold (4°C) Krebs-Henseleit (KH) physiological buffer, mounted on a modified Langendorff organ perfusion system, and perfused at a constant pressure of 10 kPa (100 cmH₂O) at 37°C with the modified KH solution (pH 7.4). The modified KH solution contained in mM: NaCl 118, KCl 4.8, CaCl₂ 1.8, MgSO₄ 0.86, KH₂PO₄ 1.2, NaHCO₃ 2.54, glucose 11 and EDTA 0.027. The KH solution was first filtered through a 0.5 µm cellulose acetate membrane and then continuously gassed with a mixture of 95% O₂ and 5% CO₂. Left ventricular developed pressure (LVDP) was monitored using a saline filled latex balloon inserted into the left ventricle by way of the left atrium and connected to a pressure transducer. EPI-ECG was recorded with two electrodes positioned one on the aorta and the other on the apex of the heart. Perfused volumes were collected manually and CF measured.

To generate ROS by electrolysis, a constant 10 mA DC was applied for 1 min. A bubble trap (placed above aorta) prevented bubbles from getting into the heart. The duration of each experiment was 35 min (Figure 2A) which began when the heart-beat was stabilized (after completion of balloon insertion and electrodes connection). The whole paradigm was

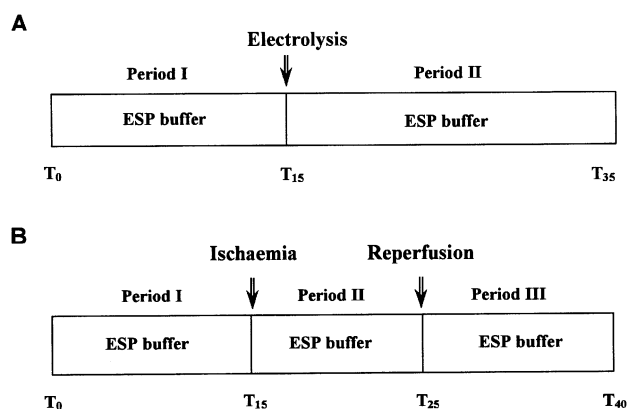


Figure 1 The experimental protocol for the isolated shark heart perfusion under the oxidative stress induced by electrolysis (A) and post-ischaemia reperfusion (B). T, time in minutes.

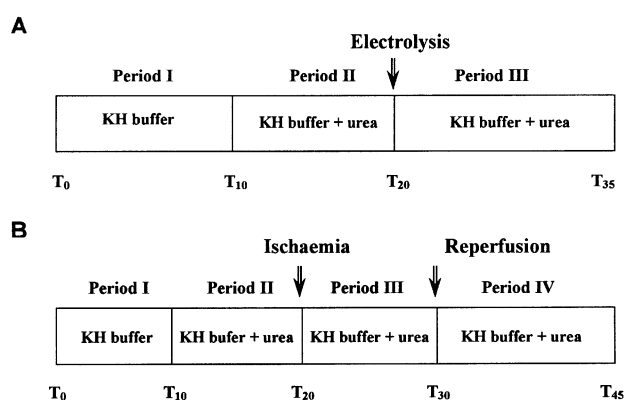


Figure 2 The experimental protocol for the isolated rat heart perfusion under the oxidative stress induced by electrolysis (A) and post-ischaemia reperfusion (B). T, time in minutes.

divided into three periods: Heart was first perfused with normal KH solution for 10 min (period I) and then with KH solution containing urea (0, 3, 30 and 300 mM) for 10 min (period II) before the start of electrolysis. The beginning of electrolysis (10 mA DC, lasted for 1 min) represented the start of period III (15 min). During this period, the heart was continuously perfused with the same urea-containing KH solution as in period II. The LVDP, CF and HR were continuously monitored during the whole experiment.

Regional ischaemia was induced by occluding the left anterior descending coronary artery by ligation as described above. The recording protocol of each experiment was set for 45 min (Figure 2B) and divided into four periods. The heart was first stabilized for 10 min with KH buffer perfusion as control (period I), then for another 10 min (period II) with KH buffer containing urea (0, 30, 150 and 300 mM). Regional ischaemia lasted for 10 min (period III) and was followed by 15 min of reperfusion (period IV) by re-opening the closed artery. The cardiodynamic parameters were continuously monitored over the whole paradigm. A typical ischaemia was marked by 40–50% reduction of LVDP and of CF accompanied by substantial elevation of left ventricular end diastolic pressure in period III, compared with pre-ischaemia values (period II). The cardioprotective effect of urea was evaluated by its ability to reduce the incidence of irreversible ventricular fibrillations (IVF) and to increase the accumulated duration of normal sinus rhythm at reperfusion during the quantification time. Arrhythmia was defined and analysed according to the Lambeth conventions (Walker *et al.*, 1988) over the first 5 min of reperfusion. Two parameters were used to quantify the arrhythmia: (a) incidence of total ventricular fibrillations which were classified into reversible (lasting for less than 2 min) and irreversible (persisting for more than 2 min) expressed in percentage of hearts presenting such fibrillations; and (b) the total accumulated time of normal sinus rhythm during the first 5 min of reperfusion.

Antioxidant effect of urea in vitro against electrolysis-induced ROS

ROS were generated by electrolyzing (10 mA, 1 min) the modified KH solution. The electrolysis cell contained 3 ml of modified KH buffer in the presence of different concentrations of urea (0, 3, 30 and 300 mM). The electrolysis-induced ROS were determined indirectly by measuring colorimetrically (Anonymous, 1985; Dumoulin *et al.*, 1996) their ability to

induce oxidation of N,N'-diethyl-p-phenylenediamine (DPD). Practically, a volume of 0.2 ml of the electrolyzed solution was added to 0.8 ml DPD reagent (25 mg ml⁻¹ in KH solution). The oxidant species react rapidly with DPD generating an oxidation product with a specific absorbency at 515 nm. The colorimetric reaction was calibrated with a potassium permanganate (KMnO₄) solution. The oxidative capacity of 1 μM of free chlorine solution corresponds to that of 0.4 μM KMnO₄ solution. The antioxidant capacity of urea was expressed as percentage of ROS scavenged. For instance, an antioxidant capacity of 100% represents the capacity of an antioxidant to scavenge the total amount of ROS generated.

Statistical analysis

Statistical significance of differences between values of LVDP, HR and CF under different treatments was evaluated with Student's *t*-test ($P < 0.05$) and the differences between the values of VF and IVF for control and treated groups were evaluated with Fisher's exact test ($P < 0.05$). All results were expressed as mean ± s.e.mean. Rat hearts were rejected if (i) LVDP < 70 mmHg; or (ii) HR < 230 beats min⁻¹; or (iii) during the period of ischaemia, the decrease of LVDP and flow rate was less than 40%. For the shark hearts, the rejection criteria were: (i) VDP < 15 mmHg; or (ii) HR < 35 beats min⁻¹.

Results

Isolated rat and shark hearts were found to have very different reactions to oxidative stress. ROS generated by electrolysis (10 mA, 1 min) induced dysfunction of the isolated rat heart: LVDP, HR and CF, measured 15 min after electrolysis, fell to 17–28% of the control values before electrolysis (Table 1, Figures 3 and 4). However, no significant dysfunction of isolated shark hearts was found under the same or even more intensive conditions of perfusate electrolyte (30 mA, 1 min) (Table 1). Exposure of isolated hearts to post-ischaemia reperfusion caused an incidence of 100% of ventricular fibrillation (VF) and 83% IVF in rat hearts while no VF was recorded for shark hearts under the same conditions. During the first 5 min of reperfusion, the time of sinus rhythm was 0.4 ± 0.2 min (8 ± 0.6% of the total time) for rat heart and 4.9 ± 0.8 min (98 ± 6% of the total time) for shark heart (Table 1).

To verify if the oxidative stress resistant properties of isolated shark hearts were related to the presence of urea or

Table 1 Reactions of the isolated rat and shark hearts to the oxidative stress induced by electrolysis and post-ischaemia reperfusion

Origin of heart	Electrolysis			Post-ischaemic reperfusion		
	LVDP (%)	CF (%)	HR (%)	VF (%)	IVF (%)	TSR (%)
Shark (n=4)	97 ± 4	100 ± 6	94 ± 5	0	0	98
Rat (n=12)	17 ± 1	24 ± 2	28 ± 2	100	83	8

LVDP, left ventricular developed pressure (VDP for shark); CF, coronary flow; HR, heart beating rate; VF, ventricular fibrillation; IVF, irreversible ventricular fibrillation; TSR, time of sinus rhythm. In electrolysis, the data measured 15 min after electrolysis were expressed as the percentage of that measured before electrolysis. TSR were expressed as the percentage of the total time of the quantification period (the first 5 min in reperfusion).

TMAO in EPS, the heart function was re-evaluated after urea or TMAO in EPS was equimolarly replaced by an inert compound, sucrose. The heart was first perfused with normal EPS (350 mM urea) for 10 min and then with the urea- or TMAO-replaced EPS for 10 min followed by 1 min of electrolysis (10 mA) and 9 min continuous perfusion.

Table 2 shows that TMAO could be replaced by sucrose without changing significantly the shark heart function ($n=4$, $P>0.05$). The resistance of shark heart to the oxidative stress induced by electrolysis was also not changed in the absence of TMAO ($n=4$, $P>0.05$). On the other hand, the replacement of urea resulted in a gradual decrease of heart beating rate and eventually arrested the heart ($n=4$). Therefore, it was not feasible to further evaluate if the shark heart perfused with the urea-free EPS was still resistant to oxidative stress. It seemed that urea was indispensable for the normal shark cardiac functions. These results also indicated that the protection

offered by urea in ESP was not due to an osmolality factor. The electrolysis of the modified EPS, in which urea was equimolarly replaced by sucrose, generated *in vitro* the same amount of ROS as the electrolysis of KH solution, while the normal urea-containing EPS only generated $6\pm 0.4\%$ of that of KH solution.

The electrolysis-induced cardiac dysfunction of isolated rat heart was decreased by the addition of urea to KH buffer. The concentration-dependent and time-dependent effects of urea on isolated rat hearts are shown in Figure 3. The relative potencies of urea at different concentrations in protecting rat heart functions from electrolysis damage are shown in Figure 4. When the electrolyzed urea-free KH solution was used, LVDP dropped to $17\pm 1\%$ of the control value obtained before electrolysis. With 3 mM of urea included in the electrolyzed KH solution, LVDP was totally recovered, and HR was increased from 28 ± 2 to $65\pm 5\%$ and CF from 23 ± 2

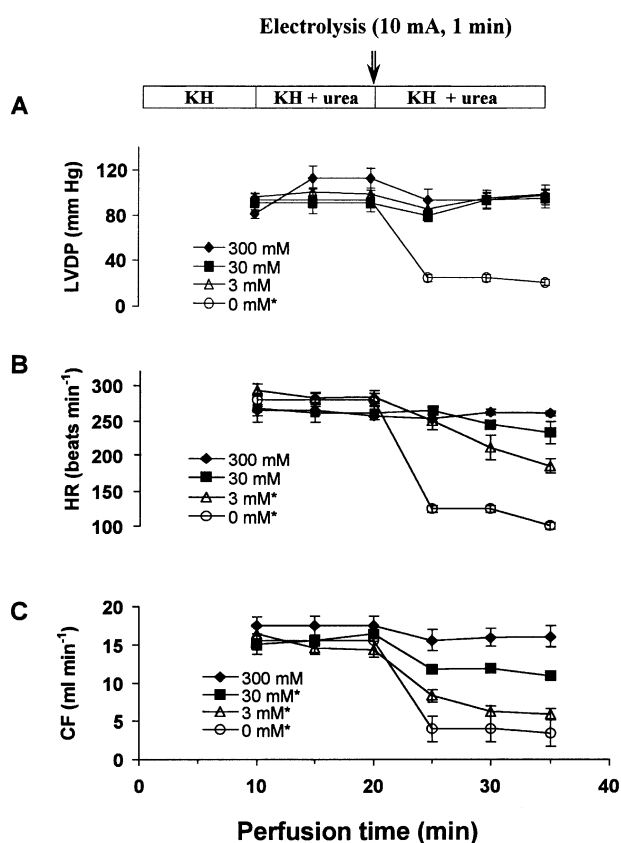


Figure 3 The concentration- and time-dependent effects of urea on the isolated rat heart. *Indicates that the data measured 15 min after electrolysis (T_{35}) were significantly different from that measured before electrolysis (T_{20}) ($P<0.01$). The concentrations (mM) shown in inserts are those of urea used in the experiments.

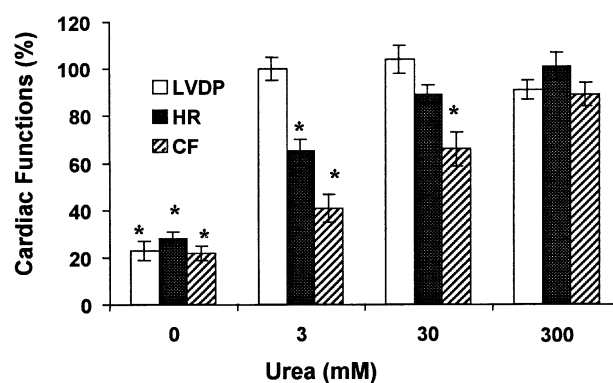


Figure 4 Relative change in cardiac functions after electrolysis damage. Left ventricular developed pressure (LVDP), heart beating rate (HR) and coronary flow (CF) of the isolated rat heart recorded 15 min after the electrolysis (10 mA, 1 min) were compared with the control values measured in the same heart before electrolysis (100%). $n=4$ for each group. * $P<0.01$ vs control.

Table 3 Antifibrillatory effect of urea and ceruloplasmin on the isolated rat heart during post-ischaemia reperfusion

Treatment	Concentration	Incidence (%)		Time of sinus rhythm (s)
		VF	IVF	
Control ($n=12$)	–	100	83	25 ± 12
CP ($n=8$)	$1\ \mu\text{M}$	33	0	263 ± 10
Urea ($n=4$)	30 mM	100	75	73 ± 63
Urea ($n=4$)	150 mM	100	50	134 ± 54
Urea ($n=4$)	300 mM	100	0	200 ± 14

VF, ventricular fibrillation; IVF, irreversible ventricular fibrillation; CP, ceruloplasmin.

Table 2 The roles of urea and TMAO in the EPS solution on the shark heart function

EPS composition	Control		Replaced with sucrose		Replaced with sucrose and with electrolysis	
	VDP (mmHg)	HR (beats min^{-1})	VDP (mmHg)	HR (beats min^{-1})	VDP (mmHg)	HR (beats min^{-1})
TMAO	17.0 ± 2.0	49.0 ± 3.4	16.0 ± 1.2	47.3 ± 3.5	16.5 ± 0.6	47.0 ± 3.6
Urea	18.2 ± 2.6	48.0 ± 2.8	0.0 ± 0.0	Arrest	N.A.	N.A.

EPS, Elasmobranch physiological solution; HR, heart beating rate; VDP, ventricular developed pressure; TMAO, trimethylamine N-oxide; N.A., not applicable.

to $41 \pm 3\%$. When 300 mM of urea was included in the electrolyzed KH solution, the recoveries of LVDP, HR and CF were 87–97% and not statistically different from the values before electrolysis ($n=4$, $P>0.05$).

In isolated rat hearts, urea had a direct cardiac effect. At a high concentration of 300 mM, urea induced an initial $59 \pm 6\%$ LVDP depression (lasted 30 s) followed by a rebounded increase of LVDP to a stable level of $35 \pm 4\%$ ($n=4$) higher than the control. Meanwhile, HR and CF remained unchanged. At concentrations equal to or lower than 30 mM, urea only induced a transient decrease (less than 1 min), but not the rebounded increase, in LVDP. Urea also protected the isolated rat heart against the damage of post-ischaemia reperfusion (Table 3). At 300 mM, urea fully protected the heart from irreversible fibrillation (IVF 0%). The cardiac protective effect of ceruloplasmin (Atanasiu *et al.*, 1995), a ROS scavenger, was also shown in Table 3 for comparison.

Our *in vitro* study showed that urea possessed an antioxidant effect against electrolysis-generated ROS in a concentration dependent mode (Figure 5). The concentrations tested were in the range from the physiological concentration of urea in human plasma (3 mM) (Guyton, 1976) to that of shark (300 mM) (Smith, 1936). The antioxidant capacities of 3 and 30 mM of urea were 56 ± 4 and $94 \pm 6\%$, respectively.

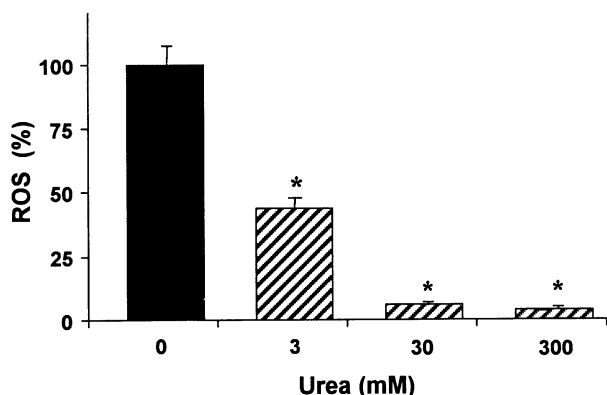


Figure 5 The relative level of ROS induced by electrolysis (10 mA, 1 min) of KH solution in the absence and the presence of different concentrations of urea ($n=4$ for each concentration, $*P<0.01$ vs control).

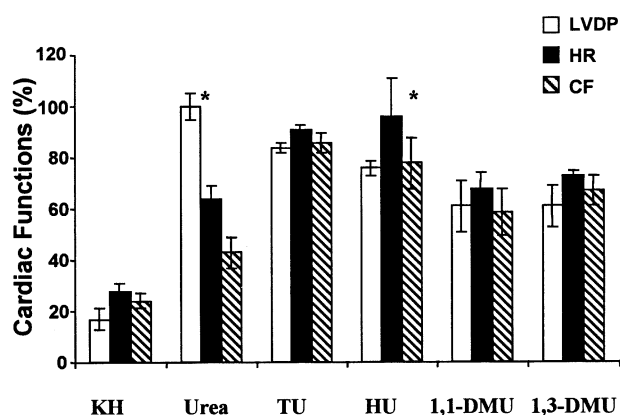


Figure 6 Cardioprotective effects of urea and urea-derivatives (3 mM) against the electrolysis (10 mA, 1 min) induced oxidative stress on isolated rat heart. TU, thiourea; HU, Hydroxyurea; 1,1-DMU, 1,1-dimethylurea; 1,3-DMU, 1,3-dimethylurea; KH, Krebs-Henseleit buffer ($n=4$ for each group. $*P>0.05$ vs control).

Table 4 Cardioprotective effects of urea and its derivatives against post-ischaemia reperfusion-induced ventricular fibrillation

Chemicals	n	Concentration (mM)	IVF incidence (%)
KH buffer	12	–	78
	4	30	75
Urea	4	150	50*
	4	300	0*
Thiourea	4	30	100
	4	150	Toxic**
Hydroxyurea	4	30	100
	4	150	75
1,3-dimethylurea	4	30	75
	4	150	75

* $P<0.05$. **Inducing significant decrease of left ventricular developed pressure.

Hydroxyurea, dimethylurea, thiourea, 1,1-dimethylurea, and 1,3-dimethylurea (N,N'-dimethylurea) at a concentration of 3 mM also had cardioprotective effects on the isolated rat hearts against the damage induced by electrolysis (Figure 6). It appears that urea derivatives were more effective than urea in protecting HR and CF. However, these derivatives failed to protect the rat heart from IVF even at 150 mM (Table 4).

Discussion

It was reported that urea could protect brain and liver tissue homogenates from lipid peroxidation (Lukash *et al.*, 1980). In this context, several interesting questions need to be answered. First, does urea protect intact isolated organs such as heart from ROS injury? Second, does the isolated heart from species with high plasma urea content, such as dogfish shark, tolerate the impact of ROS? Finally, does urea possess ROS scavenging properties? This study tentatively answered these questions.

Urea not only protects the tissue homogenates from oxidative damage, but also protects the whole organ, as shown in the present study, against oxidative stress. Cardioprotective effects of urea were tested in our study using a perfusate electrolysis model (Jackson *et al.*, 1986). This system generates a variety of OFR and ROS, allowing the evaluation of cardioprotective properties of various antioxidants (Mateescu *et al.*, 1995; Dumoulin *et al.*, 1996). Under electrolysis-induced oxidative stress, LVDP remains essentially unchanged in the presence of 30 mM of urea (Figure 3). Without urea LVDP decreased to $17 \pm 1\%$ of the control value before electrolysis. The other cardiodynamic variables, HR and CF, were also greatly protected from electrolysis damage in the presence of urea. The relative recovery of LVDP, HR and CF were not synchronic with the increase of urea concentration. For instance, 3 mM of urea fully recovered LVDP while 300 mM of urea was required for a full recovery of CF. These results are in agreement with data obtained with other antioxidants such as ceruloplasmin. In fact, it was observed that among the cardiodynamic variables, CF recovery was slower under antioxidant treatment in post-ischaemia reperfusion (Atanasiu *et al.*, 1995; Xia *et al.*, 1996; Mondovi *et al.*, 1997). It is possible that the vascular tissues and cardiac tissues may react differently to ROS. The scavenging of ROS by urea or other antioxidants could suffice to recover the contractility of ventricular muscles but the constricted coronary arteries might remain at a partial constricted status due to the existence of a smooth muscle intrinsic 'latch-bridge' contraction mechanism

(Murphy, 1994). Moreover, urea can directly decrease the affinity of pyruvate kinase for pyruvate (Burg *et al.*, 1996) which plays different roles in the energy metabolism of cardiac muscles and vascular smooth muscles. The full recovery of CF in the presence of 300 mM urea could also be explained by a direct vasodilating effect of urea at high concentrations (Vajragupta *et al.*, 1996).

The cardioprotective effects of urea are probably not related to osmolality change of the perfusate. In fact, Abaurre *et al.* (1989) showed that the change of osmolality (by adding 17 mM urea) to the perfusate did not affect the mechanical and electrical properties of the isolated rat heart but decreased the systolic ventricular pressure. In our experiments, high concentration of urea only induced a transient change in LVDP, but not HR or CF, in isolated rat hearts. As urea is virtually free to permeate cell membranes, any osmolality difference between extracellular and intracellular solutions induced by adding urea to the extracellular solution would be eliminated within a short period of time. The notion that the cardioprotective effects of urea are not due to osmolality change is also supported by the data obtained with sucrose replacing urea in EPS (Table 2).

Urea appears to protect the isolated rat heart against post-ischaemia reperfusion injury. In the presence of 300 mM of urea, IVF were completely prevented. This effect is comparable to that observed with 1–2 μ M ceruloplasmin as antiarrhythmic agent (Atanasiu *et al.*, 1995) under similar conditions.

The isolated heart from dogfish shark, *Squalus acanthias*, was chosen as model in the present study because this fish has a high plasma urea concentration of approximately 350 mM (Smith, 1936). Our results show that the isolated shark heart could withstand up to 30 mA DC for 1 min or to 10 mA DC for 3 min electrolysis of the perfusate, as well as to damage induced by reperfusion after 10 min of regional ischaemia, without any significant change of the cardiodynamic variables (Table 1). In the case of rat heart, electrolysis under milder conditions (10 mA for 1 min) and post-ischaemia reperfusion resulted in a decline of the cardiodynamic variables to 17–28% of the normal values (Mateescu *et al.*, 1997) and a 83% incidence of irreversible ventricular fibrillations (Atanasiu *et al.*, 1995), respectively. Considering that arrhythmias of rat heart induced by electrolysis and by post-schaemic reperfusion may be caused by ROS (Jackson *et al.*, 1986; Simpson & Lucchesi, 1987; Manning, 1988), the high tolerance of the shark heart to the oxidative stress is likely to be ascribed to the protective antioxidant activities of urea (350 mM) in the EPS solution.

Finally, an important antioxidant effect of urea was demonstrated. It was observed that urea concentration-dependently scavenged the ROS induced by electrolysis of the KH solution with a scavenging capacity of $56 \pm 4\%$ at 3 mM and $94 \pm 12\%$ at 30 mM.

The importance of urea in maintaining the normal function of the shark heart, the cardiac protective effects of urea on the isolated rat heart, and the ROS scavenging by urea, support the hypothesis that urea plays a role in the antioxidant defence of the organisms. This hypothesis can explain, in part, the pathogenesis of the dialysis disequilibrium syndrome (DDS) characterized by neurologic deterioration and cerebral oedema after haemodialysis of patients with end stage renal failure (Silver *et al.*, 1992). Complement-dependent granulocyte activation during haemodialysis causes the production of ROS (Himmelfarb & Hakim, 1997). The ROS are involved in the vascular injury with subsequent oedema and brain neuronal dysfunction (Demopoulos *et al.*, 1980; Chan *et al.*, 1982; Hammond *et al.*, 1985). The effects of ROS generated by

granulocyte activation and by other ROS producing systems may be limited and inhibited by high concentrations of urea. As a result of haemodialysis, antioxidant defence is rapidly decreased due to the clearance of plasma urea, resulting in the cerebral-oedema. In fact, the addition of urea into the haemodialysate to a level equal to the plasma urea level before dialysis was shown to prevent cerebral oedema during haemodialysis (Silver *et al.*, 1992). Dysfunction of the isolated shark heart when perfused with urea free EPS may be explained in the same way. The removal of urea from EPS may result in a pro-oxidant–antioxidant unbalance allowing production and accumulation of ROS that cause heart dysfunction.

As an antioxidant, urea has several advantages over many other natural or synthetic antioxidants. Due to its low molecular weight and easy passing through cell membranes, urea appears to be more mobile compared to macromolecular antioxidants such as superoxide dismutase (SOD), catalase, and ceruloplasmin, whose total antioxidant capacity appears confined by their limited mobility and their compartmental distribution. Among the main natural antioxidants, the contribution of vitamin E to the total antioxidant activity of serum is very small (Stocks *et al.*, 1974; Wayner *et al.*, 1985). The low plasma concentration (34–111 μ M) (Catherine, 1995) and the potency of vitamin C to act as a pro-oxidant (Chow, 1988) limit its contribution to the serum total antioxidant activity. Uric acid was suggested to have a biological role as an antioxidant contributing to the life span of primates with low urate oxidase activity (Seegmiller, 1979). However, serum concentrations of uric acid slightly higher than normal, due to its low solubility, are considered a risk factor of gout and heart disease (Reddy *et al.*, 1982). Considering all the facts discussed above, urea can probably be regarded as an ideal plasma antioxidant. It is worth noting that, however, urea failed to show its antioxidant activity in some conditions, such as to prevent damage induced by OFR (mainly hydroxyl radicals) in xanthine–xanthine oxidase system (Halliwell, 1978). The underlying mechanism for the different effects of urea under different conditions should be further investigated, especially in terms of its scavenging capability.

In addition, the cardioprotective capacity of urea may also be due, in part, to its activity to inhibit the production of the gaseous free radical nitric oxide, NO. It was established that the amount of NO is significantly increased upon the reperfusion of the isolated ischaemic heart (Zweier *et al.*, 1995; Wang & Zweier, 1996). There is strong evidence that NO is involved in the process of myocardial reoxygenation injury (Beckman *et al.*, 1990; Matheis *et al.*, 1992). Recently, it was found that urea was an inhibitor of nitric oxide synthase (Prabhakar *et al.*, 1997). Therefore, the cardioprotective effect of urea can be reinforced by a decreased NO production from cardiac tissues during myocardial reperfusion. However, whether urea can inhibit the basal NO formation is still open for investigation. It is also worth noting that NO can be implicated both in myocardial reperfusion injury and in the protective ischaemic preconditioning (Takano *et al.*, 1998). Nevertheless, our present study demonstrated the cardiac protecting effect of urea while it was present throughout the whole ischaemia-reperfusion process. If urea cannot be applied prior to ischaemia, a cardiac protective effect of urea applied prior to reperfusion would also be in force considering both the antioxidant effect of urea and the urea-induced inhibition of inducible NO synthase.

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